A BRIEF STUDY OF THE RANGE OF ERROR IN MICRO-ENUMERATION

BY

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Like many other people who have undertaken statistical study of microscopic organisms, I worked for a long time without any very great effort to determine the accuracy of enumeration. This was probably due to five reasons: first, various authorities state that by counting into so many hundreds or thousands, the limits of accuracy are reached, second, because two or three trial counts indicated substantial agreement, third, because the series of counts seemed to follow a normal sequence, fourth, because the insistent demands of routine work made it difficult to undertake a study of this sort, fifth, because it seemed that if great care were observed in handling and counting there was no great probability of improving matters by making such a study.

But it so happened that my co-worker in the Scripps Institution, the late Mr. E. L. Michael, when looking over the manuscript of my paper on the plankton of the San Joaquin River, raised the question as to the accuracy of my counts. We discussed the matter at various times and he always remained sceptical as to my guess that my counts were not in error more than plus or minus ten per cent. So, finally, when I got settled down to my regular program of work on marine phytoplankton, after adopting the measured water method of collecting, it became necessary to get more definite information concerning the accuracy of the counts.

I have made no thorough search of plankton literature for a record of such studies, but I have had access to the most important European and American papers, which I have scanned rather hastily without finding any indication of such a record. Hence, it seemed to me that my experience might be of some value to other workers in this or similar lines. I also thought it might lead some one to make a more thorough study of this interesting problem.

To one who has not given any serious thought to the matter, it may appear that the counting of microscopic organisms is quite similar to the counting of any common objects such as beans or apples. In the case of the plankton organisms, this is not true for several reasons. In the first place, there is usually a certain amount of dirt or débris likely to hide some individuals. Then there is the fact that if one wishes to be sure of getting a required number of the organisms, he must (because he cannot see them) filter a sufficient amount of water to give an actual excess over what he is able to count. He must then (except in the use of one or two highly specialized methods) take a fractional part of his catch and estimate the total from the number found in this fractional part. The extraction of the fractional part from the whole and the even spreading of this under the microscope for counting is an important phase in the routine of plankton counting. One can take a pint of beans and after counting the number contained, compute fairly accurately the number in a bushel or a car, but he cannot take the individual organisms one by one from his fractional measure and make such an exact estimate. Furthermore, the microscopic things are necessarily handled in fluid through which they should be nearly uniformly spread for count. If one had to take beans mixed in four or five or one thousand times their volume of water and make the count while they were in the mixture, he might have a little better idea of the difficulty of microscopic counting. Furthermore, there is the matter of eye fatigue and the difficulty of recording the count as it progresses.

A few days after beginning the work of collecting by the measured water method on September 1, 1919, I made a beginning at a study of accuracy of enumeration which I was obliged to discontinue. I did, however, make eight counts of a catch (7728) taken in the forenoon of September 6. The results of these counts are partly summarized in table I.

TABLE I

Eight counts by non standard method, of Catch 7728

No. of Count	1		2		3		4		5		6		7		8		
	Total	Per cent of Deviation	Total	Per cent of Deviation	Total	Per cent of Deviation	Total	Per cent of Deviation	Total	Per cent of Deviation	Total	Per cent of Deviation	Total	Per cent of Deviation	Total	Per cent of Deviation	Average
Diatom cells	1	91	17	40	8	33	5	41	11	8	3	25	24	100	27	150	12
Dinoflagellate cells	350	6	334	1	228	14	292	10	275	17	398	23	406	24	294	11	329
Diatom and Dinoflagellate cells	351	3	351	3	296	13	297	12	286	16	401	15	430	21	321	6	341

The series was not very good because the conditions of counting were not nearly enough alike, first because the first four counts were made on the same day and the other four at intervals of one to four days, second, because the material was kept in the mixing tube throughout the series, merely being shaken up after return of the fractional amount from the slide after each count. Furthermore, the number of diatoms was so small as to make that part of the count unreliable and none of the counts were carried quite so far as necessary to give sufficiently dependable results. In spite of these deficiencies the table shows that the series was sufficiently good to be considered statistically significant. Thus it appears that five out of the eight counts of dinoflagellates showed deviations from the mean of less than fifteen per cent and the highest per cent of such deviation was twenty-four. The showing for total numbers of cells is even better, a fact which calls attention to the general probability that a deviation of count in one group may be largely obscured by the count of another group when the two are combined for a total.

Although the table gives great emphasis to the point that the count of such a few individuals as those of the diatoms is valueless as a basis of generalization, it should not be forgotten that such a count may be worth recording because of its positive indication of presence of organisms. Furthermore, the system of random sampling to which we are usually forced, may sometimes lead to just as great differences in estimating the plankton population as is represented here. The significance of both errors becomes rapidly less with increase in numbers of samples.

Constantly harassed by the feeling that I ought to still further improve my basis of judgment as to the values of individual counts, I finally returned to a study of the problem on January 21, 1920, and gave it a large part of my time for the next two months. First I took some care in the selection of a catch for study and finally decided on the one (8102) for 8 P. M. on January 11, 1920, because it showed fairly good representation of both diatoms and dinoflagellates and also because it was relatively free from dirt. First I made ten counts of this catch at intervals of one day or more, the slide being emptied into the mixing tube each time, but the whole being left there instead of being returned to the bottle. The summary of results for this series is shown in table II.

TABLE II

Ten counts of Catch 8102

	Percentage of deviation from the mean									
Number of Count	1	2	3	4	5	6	7	8	9	10
Total Diatom Cells	29	15	10	75	28	5	10	20	2	12
Total Dinoflagellate Cells	4	18	29	3	9	2	33	15	21	18
Above totals combined	23	11	14	58	24	3	11	11	6	13

In this series a few of the more abundant organisms were only counted on one tenth or one twentieth of the slide although most species were counted on one fourth. At any rate, there is some probability that the counts of those forms which were most abundant were not carried quite far enough to yield really satisfactory results. Even so, the table shows that in only three counts out of the ten was there more than fifteen per cent deviation from the mean in total numbers of organisms and of diatoms and that there was similar deviation in only four out of the ten totals of dinoflagellates. Stated in another way, the showing is that sixty to seventy per cent of the counts deviated from the mean by not more than plus or minus fifteen per cent.

A momentary inspection of table II shows that the fourth count was the only one in which the deviation exceeded thirty-three per cent and that the enormous deviation in that case was due to some difference in the count of diatoms. Three possible causes of this great deviation

TABLE III
Two counts each of ten successive catches

Catch Number		Regular Count	Recount	Average	Per cent of Deviation
	Total Diatoms........	1112	1814	1463	24
8102	Total Dinoflagellates...	468	576	522	10
	Combined totals.......	1580	2390	1985	20
	Total Diatoms........	1572	2460	2016	22
8104	Total Dinoflagellates...	276	440	358	23
	Combined totals.......	1848	2900	2374	22
	Total Diatoms........	2098	2011	2054	2
8105	Total Dinoflagellates...	358	404	381	6
	Combined totals.......	2456	2415	2435	1
	Total Diatoms........	2898	2132	2515	15
8107	Total Dinoflagellates...	256	568	412	38
	Combined totals.......	3154	2700	2927	8
	Total Diatoms........	3298	2336	2817	17
8108	Total Dinoflagellates...	372	394	383	3
	Combined totals.......	3670	2730	3200	15

TABLE III (*Continued*)
Two counts each of ten successive catches

Catch Number		Regular Count	Recount	Average	Per cent of Deviation
8110	Total Diatoms........	1602	912	1257	28
	Total Dinoflagellates...	324	426	375	14
	Combined totals.......	1926	1338	1632	12
8111	Total Diatoms........	682	728	705	3
	Total Dinoflagellates...	164	178	171	4
	Combined totals.......	846	906	876	3
8113	Total Diatoms........	1516	1386	1451	4
	Total Dinoflagellates...	316	272	294	7
	Combined totals.......	1832	1658	1745	5
8114	Total Diatoms........	988	1652	1320	25
	Total Dinoflagellates...	424	460	442	4
	Combined totals.......	1412	2112	1762	20
8116	Total Diatoms........	2760	3348	3054	9
	Total Dinoflagellates...	158	110	134	18
	Combined totals.......	2918	3458	3188	8

require particular mention, first, it is extremely difficult to secure even distribution of the diatoms in the counting cell, second, there was an insufficient count of the more abundant diatoms, third, there may have been a personal error in keeping the tally of the count. My own opinion was that this particular deviation was mainly due to difference in the evenness of spread of the diatoms through the suspending fluid and to insufficient count.

For further test of the matter before making any very definite change in method, I then took ten consecutive catches and made two counts of each. The results are partly shown in table III.

In this table it may be noted that there was only one deviation from the mean of as much as thirty per cent. The fact that this one deviation of thirty-eight per cent was in the count of dinoflagellates might lead one to think that dinoflagellates could not be any more readily mixed through the fluid than diatoms. My detailed record of the count shows, however, that this deviation was mainly due to differences in the count of extremely minute forms which I have been including under the name Gymnodinium sp. The difficulty of seeing these forms is quite sufficient to account for this error under the circumstances. It appears, then, from this particular series that the deviation in the count of the fairly visible forms is usually well inside of thirty per cent.

In order to have some basis of judgment as to what increase in accuracy might be expected if counts were made covering the whole slide instead of a fractional part, I then made eight counts of a single catch using four different mounts. For each mount I made one count over the whole slide and one count over one fourth of the slide. The most important results are summarized in table IV.

TABLE IV
Counts of four mounts of Catch 8102

	Percentage of deviation from mean			
Number of mount	1	2	3	4
	Percentage of deviation in full slide counts			
Diatom colonies	18	5	3	25
Diatom cells	11	8	3	0
Dinoflagellate cells	5	3	3	5
Total cells	10	7	2	2
	Percentage of deviation in fourth of slide counts			
Diatom colonies	47	30	12	16
Diatom cells	30	53	25	25
Dinoflagellate cells	8	12	12	8
Total cells	25	35	20	33

While the four counts of each kind are not enough for definite conclusions, they are quite suggestive. It was not practicable to carry the series further because of the great amount of time required. As it is there is strong indication that under usual conditions the count covering the full slide is much more likely to approach the mean than is the count made over some part only.

After giving the matter a good deal of thought, I came to the conclusion that by standardizing mixing processes, much could be done toward reducing the errors of the fractional counts. I, therefore, adopted the practice of shaking the storage bottle for one minute before pouring the contents into a mixing tube, and of reversing twenty times each mixing tube used. All other manipulations had already been made as nearly uniform as possible.

I then selected for study catch number 8104 of 8 A. M., January 12, because of its close resemblance to 8102 which had become somewhat unreliable from repeated handling. Twenty counts were made of samples from this catch. At least twenty-four hours intervened between each two counts and the total catch was returned to the storage bottle after each count so that the sampling might be done in approximately the same way each time. With the first ten counts a test was made of the method of selecting fractional areas in the cell. In one case the areas were selected at intervals around the margin and in the other a median zone lengthwise of the cell and covering one fourth of its area was selected. The second ten counts were made by the median zone method but record was kept of the numbers at areas of one fifth as well as of one fourth of the slide. The results are summarized in tables V and VI.

Table V shows the percentage of deviation from the mean by marginal (twentieth to fourth of slide) and median (fourth of slide) counts in the first ten counts, calculated from the mean for this ten, by fifth and fourth of slide counts in the second ten calculated from the mean for that ten and by fourth of slide counts in the twenty counts calculated from the mean for the whole twenty. Without attempting extended analysis of the tables, I may call attention to the fact that the deviations shown by ten counts do not indicate very much difference in most cases between the marginal count (which varied from 1/20 to ¼ of the slide) and the fourth of slide count, nor between the fifth of slide and fourth of slide counts, but that there is a much greater range of deviation in the marginal counts. I also note the fact that there is a better approximation to the mean in the fourth of slide counts in the case of Gonyaulax polyedra, which is a dinoflagellate of sub-globular form. Such a difference in count of this organism might be expected because its shape would favor fairly even distribution in mixing and handling while most other organisms are sufficiently irregular in form to lead one to expect them to be more erratic in any distribution undertaken by shaking or stirring of the surrounding fluid. In the twenty count series it may be noted that the difference between Gonyaulax and total dinoflagellates tends to disappear but that the difference between both and diatoms is accentuated. The close resemblance of Gonyaulax to total dinoflagellates is attributable largely to the fact that Gonyaulax contributed about two thirds of the total.

The increased difference in range of deviation between Gonyaulax and the total diatoms is explicable on the basis of what has just been said as to differences in distribution due to form.

TABLE V—Catch 8104—Percentages of deviation from the mean

	Diatom colonies			Diatom cells			Dinoflagellate cells			Total cells			Gonyaulax polyedra		
		Median 1/4 No. of counts			Median 1/4 No. of counts			Median 1/4 No. of counts			Median 1/4 No. of counts			Median 1/4 No. of counts	
	Marginal count	10	20	Marginal count	10	20	Marginal count	10	20	Marginal count	10	20	Marginal count	10	20
1	6	9	19	1	8	16	6	6	10	0	8	16	29	7	2
2	12	12	22	17	10	18	21	3	1	12	8	16	21	3	1
3	30	13	1	15	5	4	15	10	6	49	6	3	11	1	5
4	7	0	11	9	1	10	12	7	11	10	2	11	23	14	18
5	6	1	11	12	8	1	34	12	15	16	6	3	27	3	7
6	4	3	8	0	11	1	23	3	7	3	10	0	5	16	12
7	7	12	21	0	16	24	0	8	3	0	14	21	13	7	11
8	24	14	23	37	29	36	0	10	13	33	27	33	5	12	16
9	7	5	16	11	15	23	4	7	2	10	12	20	5	5	0
10	31	38	23	35	56	42	15	9	5	28	50	37	19	5	0
	Median 1/5 of slide	10		Median 1/5 of slide	10		Median 1/5 of slide	10		Median 1/5 of slide	10		Median 1/5 of slide	10	
11	0	1	10	14	15	26	4	5	10	13	14	24	13	12	17
12	2	1	10	4	1	8	6	0	3	4	1	6	18	7	3
13	13	12	3	6	7	0	10	2	6	4	6	1	10	2	6
14	7	5	17	9	1	10	5	0	3	7	1	9	1	2	2
15	8	4	15	3	2	7	5	6	2	3	2	6	4	4	0
16	11	10	21	12	5	14	12	13	9	10	3	12	1	7	3
17	8	13	4	1	6	3	5	3	1	2	5	2	16	13	9
18	5	3	8	0	4	4	4	0	4	0	4	4	8	2	6
19	13	6	18	19	8	17	4	10	14	18	8	17	1	7	12
20	5	5	17	4	8	0	10	6	9	2	6	2	13	10	13

Table VI covers some of the same ground as table V but in a different way. In this table enumeration totals are shown instead of percentages, with the addition of a list of numbers of Gonyaulax polyedra in each of the twenty counts and a list of numbers of both cells and colonies of Nitzschia seriata in each of the twenty counts. It also includes a statistical summary which the late Mr. E. L. Michael very kindly prepared for me. The series is too short for statistical treatment but the summary has some interest in a suggestive way.

This summary indicates that the extreme deviation is not only more than twice as great in the case of diatom cells as it is in the case of Gonyaulax and total dinoflagellates but that the same thing is true of both cells and colonies of Nitzschia seriata, the most abundant diatom in the catch. Nitzschia seriata is a slender spindle-shaped diatom occurring very largely in colonies of two to six individuals. Its form would lead me to expect it to be quite erratic in distribution by any possible method of mixing. This is also to be expected of the other numerous diatoms, which belong mainly to the Chaetoceras group. It is also interesting to note that the wide range of deviation in diatoms is due to the tenth and eleventh counts and that in count ten the numbers of both dinoflagellates and Gonyaulax are very close to the mean, though Gonyaulax approaches the extreme deviation in the eleventh count.

This last point is important because of its indication that the error lies in the mixing and distributing of the organisms rather than in the method of counting. The normal count of the less erratic Gonyaulax indicates that there was no serious mistake in counting, computing or recording, while the known erratic distribution of the diatoms does indicate considerable variability in results of mixing. In spite of the large extreme deviation due to diatoms, the mean variability for total cells is only 12.2%, a fact which gives ground for thinking that totals of most counts are within a range of error of less than ten per cent.

A point which can be verified by the reader in table VI, but not in the others (though true of all), is that the deviations are fairly evenly distributed on both sides of the mean. This is an indication in this type of study that the fluctuations are normal and that they appear approximately according to expectation.

Although this study as a whole is distinctly brief and fragmentary it seems to give a good practical basis for the following provisional conclusions: First, that by very great care the extreme deviation (in total numbers of diatoms and dinoflagellates) could probably be kept within twenty-five per cent; second, that the mean deviation can be easily kept within ten per cent; third, that diatoms are more variable in the counts than dinoflagellates; fourth, that the causes of variability are to be found in the processes of mixing, sampling and spreading on the slide, rather

TABLE VI

Catch 8104

Enumeration totals, deviations, etc.

Count	Total Diatom Colonies	Total Diatom Cells	Total Dinoflagel-late cells	Total Cells	Gonyaulax polyedra	Nitzschia seriata
1st	696	2152	304	2456	220	Col. 280 Cells 660
2nd	676	2100	332	2432	212	188 372
3rd	872	2464	356	2820	204	292 560
4th	768	2300	300	2600	176	232 436
5th	764	2532	284	2816	200	252 604
6th	792	2604	312	2916	240	220 552
7th	680	1948	348	2296	192	160 372
8th	664	1652	292	1944	180	240 536
9th	728	1984	344	2328	216	264 424
10th	1060	3644	352	3996	216	308 648
11th	948	3232	368	3600	252	304 796
12th	952	2770	348	3118	208	264 532
13th	840	2596	356	2952	228	232 508
14th	1008	2828	348	3176	220	224 384

Table VI (*Continued*)
Catch 8104
Enumeration totals, deviations, etc.

Count	Total Diatom Colonies	Total Diatom Cells	Total Dinoflagel-late cells	Total Cells	Gonyaulax polyedra	Nitzschia seriata
15th	992	2760	328	3088	216	336 608
16th	1048	2940	304	3244	208	316 632
17th	832	2640	340	2980	196	252 564
18th	932	2676	348	3024	228	284 556
19th	1016	3016	384	3400	240	304 738
20th	1008	2592	368	2960	244	304 616
Extreme deviation	246=30.2%	1059=41%	52=15.5%	1089=37.4%	39=18.2%	103=39.1% 242=43.7%
Average	814	2585	336	2907	215	263 554
Standard deviation	142	463	27	410	20	44 112
Average deviation	124=15.2%	358=13.9%	23=6.9%	354=12.2%	16=7.5%	37=14% 87=15.7%

than in the counting; and fifth, that the range of error in counting is at worst far less for microplankton material than is the range of error in locating, catching and preserving material.

It seems fair to regard these results as suggestive for microscopic material in general, e. g., enumeration of blood corpuscles might be expected to show a range of error somewhat similar to that of Gonyaulax and direct enumeration of bacilli to give results, resembling those from diatoms.

As regards my own use of the study, I may say that it has led me to decide on the mixing procedure already mentioned, and in counting to carry all enumerations to fifty individuals (or fifty colonies) or to a very close approach to fifty at a convenient computing point, except that all enumerations are stopped when one eighth of the slide has been covered.

I may say frankly that for a single count or for a very short series of counts, this number limit and area limit are too small. But in handling large numbers of catches in large series and working through long periods of time, one must give close attention to the law of diminishing returns. Would the counting of a larger number of abundant forms or the counting of all over a larger area give enough greater approach to accuracy to compensate for the greater effort and use of time? It has not seemed to me that it would for present purposes. With the lens combination on a monocular microscope which was used in making this study, it was convenient to work over the area of one fourth of the slide. Later when using a different lens combination on a binocular microscope, it was found that an area of one eighth of the slide was more convenient. In fact some counts are so fatiguing and so time consuming at one fourth slide as to be impracticable in a long series. With my present standardized procedure I should expect the one eighth slide counts to show about the same range of error as indicated for the one fifth slide counts in table V. I have not yet had time to verify this assumption. At worst the range of error in careful work will certainly not be as great as that due to other factors as far as microplankton is concerned.

Finally, I may say that although the results which I have obtained are inadequate for definite conclusions, they do indicate that with standardized procedure the microscope phase of plankton study is much more nearly accurate than some of the other phases.